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**13. ABSTRACT (Maximum 200 Words)**

DNA repair via the homologous recombination (HR) pathway requires the recombinase RAD51 and, in vertabrates, five RAD51 paralogs. The paralogs form two complexes in solution, a XRCC3/RAD51C heterodimer and a RAD51B/RAD51C/RAD51/XRCC2 heterotetramer. Mutation of any one of the five paralogue genes prevents subnuclear assembly of recombinase at damaged sites and renders cells 30-100 fold sensitive to DNA cross-linking drugs. Here we used phage display to isolate peptides that bind the paralog XRCC3. Sequences of binding peptides showed similarity to residues 14-25 of RAD51C protein. Point mutations in this region of RAD51C altered its interaction with both XRCC3 and RAD51B in a two-hybrid system. A synthetic peptide composed of residues 14-25 of RAD51C fused to a membrane transduction sequence (PTD4) inhibited subnuclear assembly of RAD51 recombinase and sensitized Chinese hamster ovary (CHO) cells to cisplatin when added to growth medium. These results suggest that residues 14-25 of RAD51C contribute to a 'hot spot' utilized in both XRCC3-RAD51C and RAD51B -RAD51C interactions. Peptide-based inhibition of HR may prove useful for improving the efficacy of existing cancer therapies.

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## Table of Contents

INTRODUCTION:

We proposed to isolate peptide inhibitors of the recombinational repair protein Xrcc3p using phage display methods and test the ability of these inhibitors to enhance the effectiveness of the chemotherapeutic cis-platinum. This idea is based on genetic experiments that show cells defective in the *XRCC3* gene are 50-100 fold more sensitive to the cytotoxic effects of cis-platinum than normal cells. Preliminary tumor xenograft experiments also suggest that the effectiveness of cis-platinum treatment is greatly enhanced if tumors lack *XRCC3* function. An important connection between the function of the *XRCC3* gene and the breast cancer susceptibility gene *BRCA1* provides additional evidence indicating potential benefit from the proposed work. Genetic and cytological analysis suggest that *XRCC3* and *BRCA1* play related roles in Rad51-mediated recombinational repair of cis-platinum induced DNA damage; both genes required for the subnuclear assembly of Rad51 protein in response to cis-platinum treatment. A recent clinical study showed a significant correlation between *BRCA1* defects and favorable clinical outcome following cis-platinum treatment, a result that appears to be analogous to the results of our tumor xenograft experiments. These findings suggest combination of an appropriate recombinational repair inhibitor with cis-platinum will dramatically improve breast cancer treatment outcome compared to treatment with cis-platinum alone.

We proposed to isolate peptides that bind tightly to Xrcc3p using phage display. A library of filamentous phage displaying random 7 residue peptides was selected by Xrcc3p binding. DNA sequence analysis was used in combination with phage ELISA assays to determine a consensus peptide sequence associated with Xrcc3p binding. Peptides were synthesized that contain the consensus Xrcc3p binding sequence fused to a peptide that promotes penetration of cell membranes. Fusion peptides were tested for their ability to sensitize the breast cancer cell lines MCF-7 and MDA-321 to the lethal effects of cis-platinum treatment. Peptides were also tested in clonogenic survival and tumor xenograft assays. While these peptides may themselves be amenable to use in clinical trials, it is more likely that they will provide a starting point for future rational drug development.

## BODY:

**Statement of Work:** ***The statement of work in the orginal proposal contained 6 tasks. These tasks were exactly as listed below. The progress to date on each task is indicated.***

Task 1: Purify 1 mg of Xrcc3p from *E. coli* cells (months 1-6)

This task was completed. While purification of native protein proved impossible because of its insolubility in conventional buffers. We were able to purify denatured protein from insoluble inclusion bodies. This was accomplished by modifying the sequence of the cDNA to create a "tag" of 6 histidine residues at the amino terminus of the protein. Protein expressed in E. coli using a phage T7 expression system, denatured with 8M urea, and purified by conventional metal affinity chromatography methods. The protein was then partially refolded by removal of urea via stepwise dialysis. Yields of protein are on the order of 10mg/liter starting bacterial culture and the purity is estimated to be at least 95%.

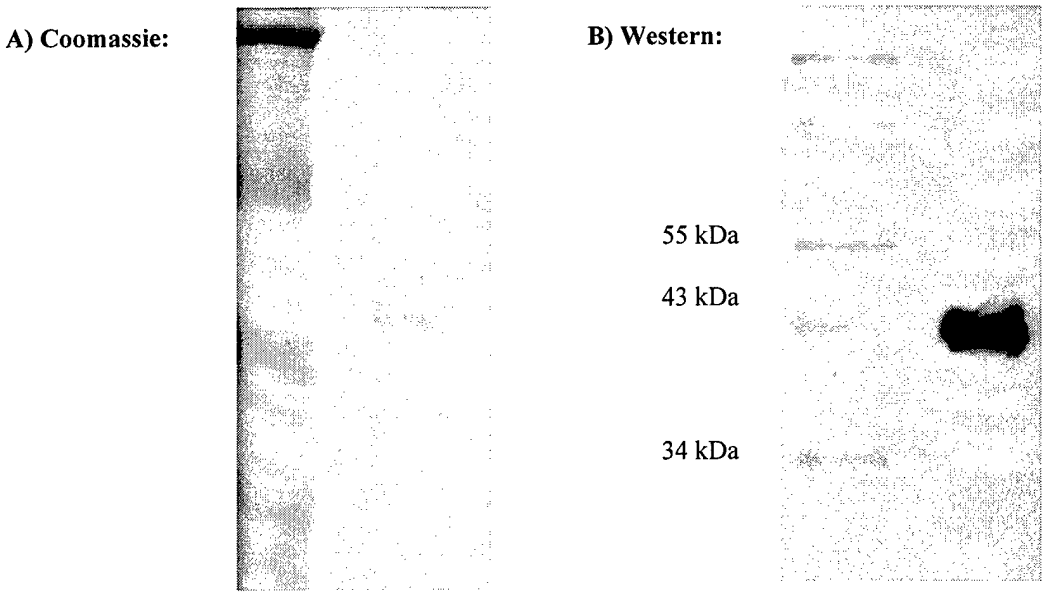



**Figure1:** Coomassie stained acrylamide gel and western demonstrating purified Xrcc3 protiein.

Task 2: Carry out initial bio-panning experiments, obtain DNA sequences of peptide coding regions, and determine consensus sequences (months 7-12).

This task was completed. Screening of random peptide phage display libraries has become a valuable method for developing peptides that bind to particular targets. Interestingly, the peptide motifs resulting from such screens can mimic or identify natural ligands of the target in question. Based on these findings, XRCC3-binding peptides were isolated with the goal of inhibiting HR by blocking protein-protein interactions. An M13 phage display library was used, wherein each phage virion expresses five copies of a random 12-residue peptide. Peptides were selected based on binding to purified 6xHis-XRCC3 and were eluted with anti-XRCC3 polyclonal antibodies. Two consecutive rounds of selection were performed. After the second round, 50 phage were sequenced to identify displayed peptides.

Task 3: Construct custom phage display libraries and carryout bio-panning experiments to optimize peptide Xrcc3p binding (12-30).

This task was deemed unnecessary based on our success with the peptide Rad51C (see below).

Task 4: Measure affinities of phage via ELISA assay, obtain DNA sequences of phage, and order synthesis of candidate peptides (months 12-30).

Twenty-eight of the XRCC3-binding peptide sequences contained known polystyrene-binding motifs (e.g. WW or WH) and were eliminated from subsequent analyses. Analysis of the

22 remaining sequences revealed 4 that could be aligned to residues 14-25 of RAD51C protein (Figure 2A). The alignment was found to be significant ($p = 0.0025$) using a multiple sequence alignment tool (MACAW, BLOSUM-80 matrix) and a search space that included full length RAD51C and the four peptides. The same analysis did not generate significant alignments when the 22 peptides were compared against other proteins (RAD51, XRCC2, XRCC3, RAD51B, RAD51D, Ku, or BSA).

**A)**

| Phage clone# | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 29 | | | | **F** | **P** | W | **S** | Q | S | H | H | Q | A | S | L |
| 43 | | | A | Y | **P** | **L** | **S** | L | I | S | **R** | A | P | P | |
| 59 | | T | S | **S** | L | **P** | K | L | **P** | F | A | P | E | | |
| 69 | | **L** | P | Y | **F** | S | **L** | **S** | **P** | D | P | F | A | | |
| **Rad51C (14-25)** | | **L** | **V** | **S** | **F** | **P** | **L** | **S** | **P** | **A** | **V** | **R** | **V** | | |

**B)**

| | RAD51C - Derived | PTD4 |
|---|---|---|
| RAD51C(14-25)-PTD4: | LVSFPLSPAVRV | – GGG – YARAAARQARA |
| RAD51C(scram)-PTD4: | SVVAPLLSRFVP | – GGG – YARAAARQARA |

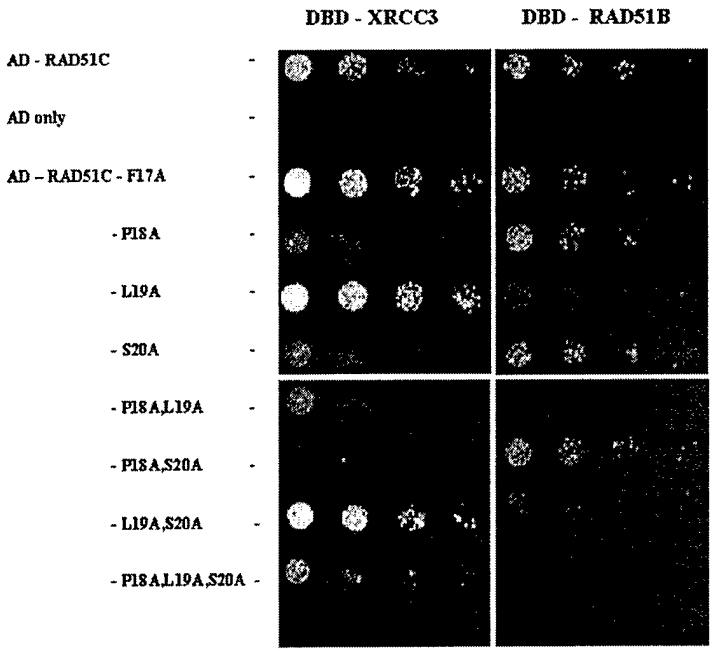
**Figure 2. Peptide sequences and alignments.** **A)** Alignment of XRCC3-binding peptide sequences to RAD51C (amino acids 14-25). Residues in bold = identity. **B)** The sequences of the synthetic peptides. A three-residue glycine linker connects the RAD51C-derived and peptide transduction segments.

RAD51C protein is known to interact with XRCC3 and RAD51B. Detection of residues in RAD51C that aligned with those in the peptides raised the possibility that these RAD51C residues might be important for XRCC3 binding and perhaps RAD51B binding as well. Yeast two-hybrid experiments were performed to test these possibilities using alanine substitution mutations in RAD51C. The two hybrid system used allows two types of assays, a qualitative growth assay in which the two hybrid interaction is required for histidine prototrophy, and a quantitative β - galactosidase assay in which the two hybrid interaction is required for expression of a $P_{GAL4}$-*lacZ* fusion gene. Two of the RAD51C mutations, P18A and S20A, resulted in reduced XRCC3 interaction in the growth assay (Figure 3A), and this corresponded to modest reductions in the β-galactosidase assay (Figure 3B). A double mutant containing both of these alanine substitutions (P18A,S20A) yielded a dramatic effect in the growth assay and a 38.6% reduction in interaction on the β -galactosidase assay. These results are consistent with a recent study by Shibata and coworkers, in which N-terminal deletions of the first 6 residues of RAD51C had no significant effect on binding to XRCC3, but deletions of ≥19 residues reduced binding. The L19A mutant, and double mutants that included L19A, displayed a strong reduction in RAD51B-RAD51C interaction in both assays. The importance of the RAD51C region for binding to both XRCC3 and RAD51B may explain why RAD51C is the only component shared by both the two paralog complexes; XRCC3-RAD51C binding and RAC51B-RAD51C binding may be mutually exclusive. Surprisingly the L19A mutation, while reducing RAD51B-RAD51C interaction, increased XRCC3-RAD51C interaction. This result suggests L19 is specifically important for RAD51C-RAD51B interaction. Specific differences between the XRCC3-RAD51C interface and

the RAD51B-RAD51C interface could provide a means to regulate the relative abundance of the XRCC3/RAD51C and RAD51B/RAD51C/RAD51D/XRCC2 complexes, or allow for dynamic changes in RAD51C interactions during the progression of DNA repair events.



3A)




3B)

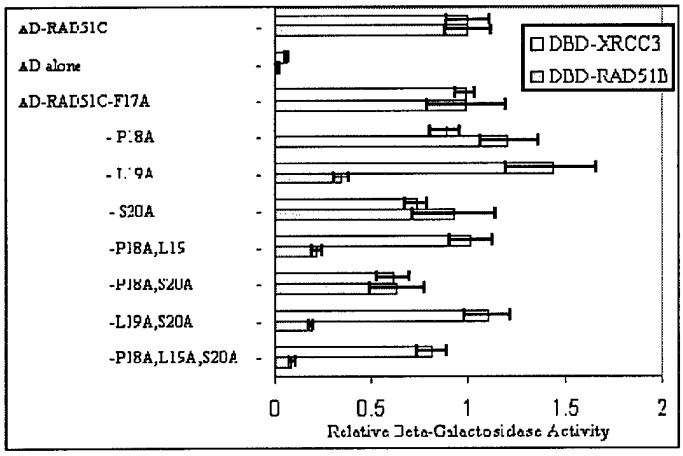


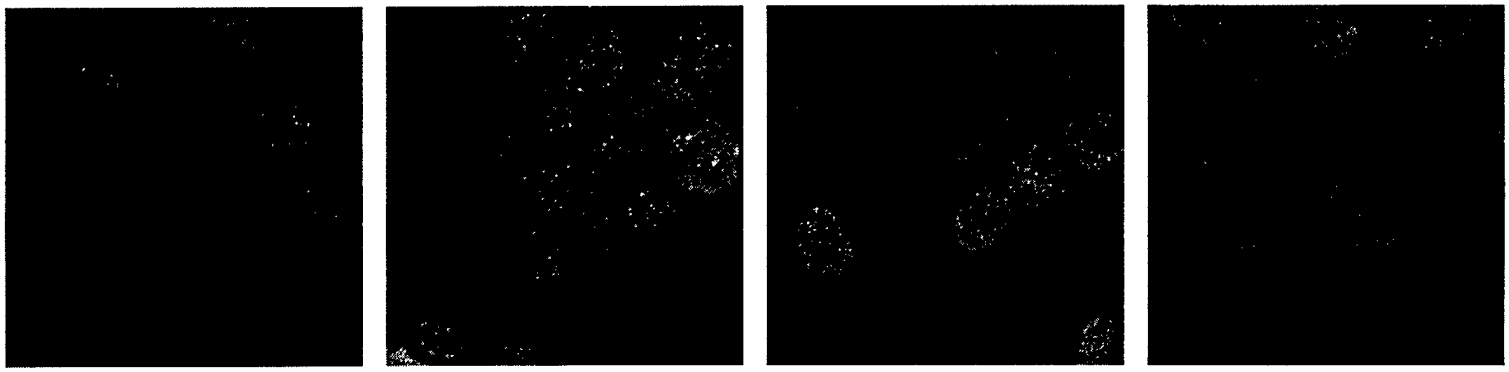

**Figure 3. Yeast two-hybrid results.** Activation domain (AD) fusions are wild type or mutant versions of RAD51C protein fused to the Gal4 activating domain. These were examined for interaction with full length XRCC3 or RAD51B protein fused to the Gal4 DNA binding domain (DBD). Western blotting of whole cell yeast extracts confirmed that each of the mutant RAD51C-AD fusions produced approximately the same steady state level of full-length fusion protein (data not shown). **A)** A five-fold dilution series was prepared from Co-transformants, plated on histidine omission medium containing 5mM aminotiazol, and allowed to grow for 2 days (DBD-RAD51B) or 5 days (DBD-XRCC3). **B)** Reporter gene expression level was determined based on beta-galactosidase activity. Cells were permeablized with Y-PER yeast protein extraction reagent (Pierce) and incubated at 30° with the chromogenic substrate o-nitrophenyl-b-galactoside (ONPG). The β-galactosidase results are pooled from two experiments (each done in triplicate) performed on separate days. The raw β-galactosidase activity was 4.1 fold greater for the wild type RAD51C-RAD51B interaction relative to wild type RAD51C-XRCC3 interaction. Alanine mutagenesis results have been normalized to the corresponding wild type interaction.

Several pure Xpb's have been obtained either by purification after expression in E. coli or by conventional peptide synthesis. We used ELISA assays to compare the relative affinities of different phage, but do not have reliable measurements of binding affinities at present because we are unsure as to the fraction of XRCC3 protein that is in a folded state. To date we have produced ~10 candidate Xbp peptides, either by direct synthesis or by expression/purification from E. coli. However, the only peptide found to be active has been the RAD51C consensus sequence itself. This RAD51C peptide sequence was synthetically prepared (Figure 2B), and was constructed in fusion to a poly-glycine linker and a C-terminal protein transduction domain (YARAAARQARA). This transduction sequence, termed PTD4, has been shown to cross lipid bilayers and promote intra-cellular drug delivery. This fusion peptide is hereafter referred to as RAD51C(14-25)-PTD4. A second peptide was also synthesized, wherein the RAD51C-containing sequence was scrambled (SVVAPLLSRFVP). The resulting fusion peptide, referred to as RAD51C(scram'd 14-25)-PTD4, served as a negative control in all subsequent experiments.

Task 5: Measure sensitivity of breast cancer cell lines to the combination of cis-platinum and Xrcc3p binding peptides in clonogenic and tumor xenograft assays (20-36).

The RAD51C(14-25)-PTD4 peptide is hypothesized to function by binding XRCC3 protein and consequently inhibiting HRR by preventing assembly of the recombinase Rad51 at sites of damage. To test this hypothesis, RAD51 focus formation assays were performed. Cells were exposed to cisplain, fixed, and then stained with anti-Rad51 antibodies to identify sites of sub-nuclear RAD51 assembly. For these experiments, XRCC3-expressing cells (irs1SF + pXR3 + pCB6) were damaged with cisplatin and then incubated with peptide. Examination of cells via confocal microscopy demonstrated that the RAD51C(14-25)-PTD4 peptide inhibited the formation RAD51 focus formation. As expected, the scrambled control peptide had no appreciable effect on focus induction. (Figure 4 a-b)

Figure 4a)



No Treatment | Cisplatin | Cisplatin + RAD51C(scram'd 14-25)-PTD4 | Cisplatin + RAD51C(14-25)-PTD4

Figure 4b)

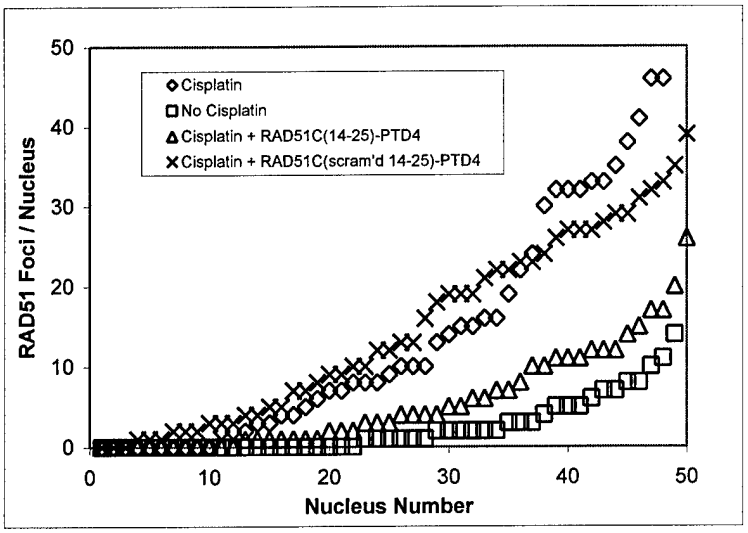



**Figure 4. RAD51C(14-25)-PTD4 inhibits sub-nuclear assembly of RAD51 following DNA damage.** XRCC3-expressing CHO cells (irs1sf + pXR3 + pCB6) were treated with 50 uM cisplatin for one hour, incubated with peptide for 3 hours, typsinized, and fixed with PFA. Cells were then transferred to microscope slides and indirectly immunostained for RAD51 (**A**). Fifty randomly selected nuclei per treatment group were examined. Within each treatment group, nuclear RAD51 focus counts were arrayed in increasing order; then the focus count observed in each nucleus was plotted as a function of its position in the array (**B**).

A synthetic fusion peptide (RAD51C(14-25)-PTD4) was prepared containing residues 14-25 of RAD51C, a three-residue poly-glycine linker, and the protein transduction domain PTD4.(Figure 2b) Peptides containing PTD4, have been shown to cross lipid bilayers and promote direct intracellular transduction of peptides and proteins. A negative control peptide (RAD51C(scram)-PTD4) was synthesized wherein the order of residues 14-25 of RAD51C was scrambled. Colony forming assays were performed to determine survival of CHO cells following cisplatin treatment in combination with peptides (Figure 5). The RAD51C(14-25)-PTD4 peptide sensitized cells to cisplatin with peptide concentrations as low as 125 nM, reducing the yield of viable clonogenic cells approximately 2-fold relative to treatment with cisplatin alone or cisplatin with scrambled control peptide (Figure 5A,B). In the absence of cisplatin, neither RAD51C(14-25)-PTD4 nor the scrambled control peptide was toxic at concentrations up to 2 μM, indicating that the killing effect of the peptide depends on cisplatin-induced damage. In contrast to its effects on CHO cells that express all five paralogs, RAD51C(14-25)-PTD4 peptide did not enhance the cisplatin sensitivity of a genetically-matched XRCC3-deficient CHO cell line (Figure 5C). This result indicates that sensitization depends on functional HR. The activity of the peptide was also tested on three human breast tumor lines MCF7, BT20, and MDA MB-231. To date, no activity has been detected in these line and efforts are underway to isolate improved inhibitory peptides.

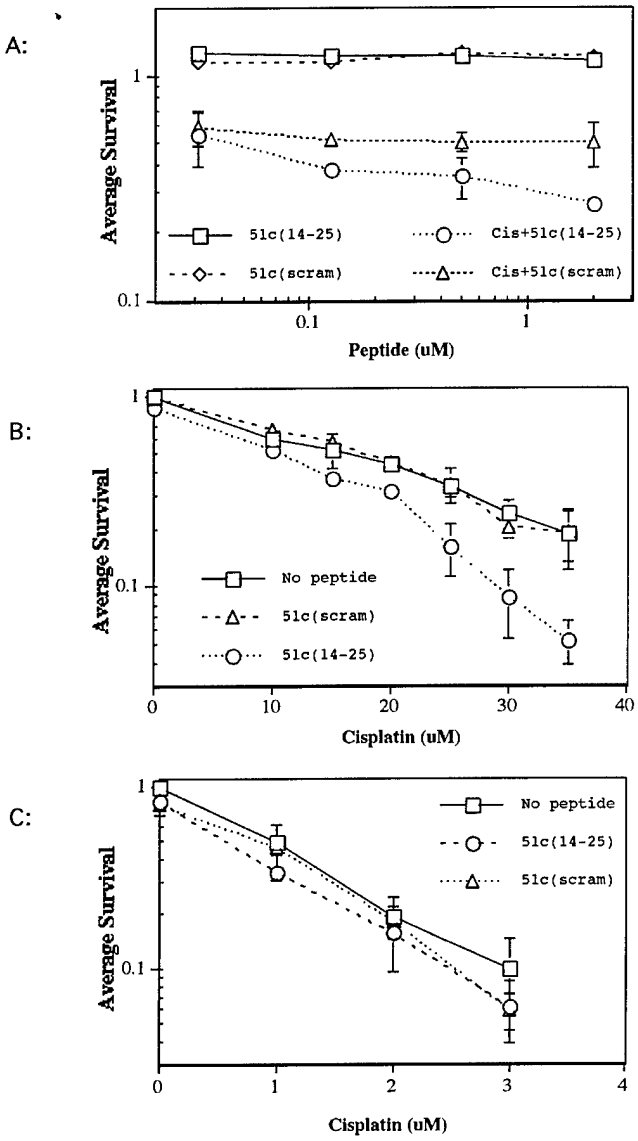



**Figure 5. Clonogenic survival assays.** Cells were plated, grown overnight, treated with cisplatin in serum-free media for 1 hour, and washed with serum-free medium. Cells were then incubated in peptide-containing media (Dulbeccos's modified eagle's medium, 10% heat-inactivated fetal bovine serum) for the remainder of the experiment (10-12 days). **A)** XRCC3-expressing CHO cells (irs1SF + pXR3 + pCB6) were treated with media containing 25 uM cisplatin (or media alone) followed by varied concentrations of peptide. **B)** XRCC3-expressing CHO cells (irs1sf + pXR3 + pCB6) were treated with varied concentrations of cisplatin followed by media containing 0.5 uM peptide. **C)** XRCC3-deficient CHO cells (irs1sf + pCB6) were treated with varied concentrations of cisplatin followed by incubation in 0.5 uM peptide.

Xenograft experiments were performed to confirm the efficacy of our peptide in this tumor model. *XRCC3* rescued CHO cells (irs1SF + pXR3 + pCB6) were injected subcutaneously into nude mice and were allowed to form tumors. Histologic examination of the resulting tumors demonstrated a resemblance to human fibrosarcomas (data not shown). After 10 days for tumor growth, the mice were treated with cisplatin (10 ug/day intra-peritoneal) for 5 consecutive days or PBS as a control. Tumors were also treated via dialy intra-tumoral injection of peptide. In one initial experiment, the combined treatment with RAD51C(14-25)-PTD4 and cisplatin (35 ug/day intra-peritoneal) resulted in enhanced tumor shrinkage, relative to cisplatin + scrambled control peptide. However, when repeated on 3 subsequent experiments, this effect could not be repeated. No animal death or significant toxicity was observed in any of the treatment groups.

Task 6 (if Task 5 does not yield positive results ): Construct expression plasmids that direct high level expression of Xrcc3p binding peptides, transfect plasmid into breast cancer cell lines, and measure the sensitivity of transfectants to cis-platinum (months 31-36).

To date, all successful peptide-mediated sensitization to cisplatin has been in CHO cells. All attempts using human breast cancer cell lines have been unsuccessful. We are confident that the peptides are successfully penetrating into human breast cells following direct transduction with the PTD4-conjugated tags. For this reason we have not pursued methods achieving intracellular peptide expression with plasmids. Instead, we have begun working toward developing improved peptides with higher binding affinities to XRCC3.

Specifically, we have begun to develop peptides that bind the RAD51C/XRCC3. The RAD51C/XRCC3 protein complex has been purified, plated on microtiter wells, and subjected to the phage display process. We are employing a variety of non-specific and specific elution conditions to select for peptides that strongly bind the RAD51C/XRCC3 complex.

As as alternative, we have begun high-throughput optimization of peptide sequences in order to maximize binding affinity to RAD51C/XRCC3. In this system, oligonucleotide duplexes encoding peptide sequences are cloned into an AP expression plasmid. The resulting peptide-AP fusion proteins are purified, and their binding affinities to RAD51C/XRCC3 are assayed based on quantification of AP activity. This system allows for a large number of peptide sequences to be efficiently screened at a low expense. For any successful peptide, this method will first be used to analyze other closely related peptides (10-20) that were found during the phage display process. Once the strongest binder within such a group has been identified, alanine substitution mutants will be prepared for each of the12 residues in a candidate peptide to determine which residues are important for binding to RAD51C/XRCC3. For amino acid positions determined to be non-essential for binding, random mutagenesis will then be performed in an attempt to improve upon binding affinities. These AP-fusions will also be compared against one another for their ability to inhibit DNA binding. Since this AP-fusion system is only a semi-quantitative assay, plasmon surface resonance technology (BIACore) will also be used to quantify the binding constants of selected synthetic peptides and AP-fusions. BIACore is very sensitive to small differences in binding affinities, and this will help chose the optimal peptides from a group of relatively strong binders. Hopefully, binding constants in the low nM range can be achieved with this optimization strategy.

## KEY RESEARCH ACCOMPLISHMENTS:

- XRCC3 protein was expressed and purified.
- Several peptides that bind XRCC3 have been isolated following phage display selection.
- XRCC3 binding peptides resemble a portion of RAD51C (amino acids 14-25)
- RAD51C(14-25)-PTD4 peptide sensitizes cells to cisplatin and inhibits sub-nuclear assembly of RAD51 following DNA damage.

## REPORTABLE OUTCOMES:

- XRCC3 binding peptides resemble a portion of RAD51C (amino acids 14-25)
- These results suggest that residues 14-25 of RAD51C contribute to a 'hot spot' utilized in both XRCC3-RAD51C and RAD51B -RAD51C interactions.
- RAD51C(14-25)-PTD4 peptide sensitizes cells to cisplatin and inhibits sub-nuclear assembly of RAD51 following DNA damage.

Manuscripts, abstracts, presentations:

- Poster presentation at the D.O.D. Era of Hope meeting, Orlando, September 2002.
- Oral presentation at A.A.C.R. meeting, Washington, July 2003.
- Manuscript submitted and in review.

Patents and licenses applied for and/or issued:
Provisional patent application was filed for RAD51C(14-25)-PTD4 and related peptides.

Degrees obtained that are supported by this award; none

Development of cell lines, tissue or serum repositorie resulting from the award: not applicable

Infomatics such as databases and animal models, etc.; not applicable.

Funding applied for based on work supported by this award:

Department of Defense, Idea Development Award "Targeted Inhibition of DNA repair in Prostate Cancer"
Dr. Ralph Weichselbaum, P.I. (funded) Start Date 1/1/2002.

Department of Defense, Prostate New Investigaror, "SENSITIZING PROSTATE CANCER CELLS TO CHEMOTHERAPY AND RADIATION VIA INHIBITION OF NON-HOMOLOGOUS END-JOINING DNA REPAIR"
Dr. Philip Connell, PI (not funded)

Department of Defense, Breast Idea Development Award, "SENSITIZATION OF BREAST CANCER CELLS TO RADIATION AND CHEMOTHERAPEUTIC AGENTS VIA INHIBITION OF HOMOLOGOUS RECOMBINATIONAL DNA REPAIR."
Dr. Douglas Bishop, PI (presently under review)

NB: there is no overlap between the aims of either of these proposals and the proposal being reported on here.

Employment or research opportunities applied for and/or received based on experience/training supported by
this award.:none as of 9/30/01

CONCLUSIONS:

DNA repair via the homologous recombination (HR) pathway requires the recombinase RAD51 and, in vertabrates, five RAD51 paralogs. The paralogs form two complexes in solution, a XRCC3/RAD51C heterodimer and a RAD51B/RAD51C/RAD51/XRCC2 heterotetramer. Mutation of any one of the five paralogue genes prevents subnuclear assembly of recombinase at damaged sites and renders cells 30-100 fold sensitive to DNA cross-linking drugs. Here we used phage display to isolate peptides that bind the paralog XRCC3. Sequences of binding peptides showed similarity to residues 14-25 of RAD51C protein. Point mutations in this region of RAD51C altered its interaction with both XRCC3 and RAD51B in a two-hybrid system. A synthetic peptide composed of residues 14-25 of RAD51C fused to a membrane transduction

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P. P. Connell, N. Siddiqui, S. Hoffman, A. Kuang, R. R. Weichselbaum, D. K. Bishop, Proceedings of the Era of Hope Meeting, Orlando, 2002.

P. P. Connell, N. Siddiqui, S. Hoffman, A. Kuang, R. R. Weichselbaum, D. K. Bishop, Proceedings of the AACR Meeting, Washington, 2003.

APPENDICES:

not applicable.